AD_____

Award Number: DAMD17-01-1-0124

TITLE: Selenium and Breast Cancer Chemoprevention

PRINCIPAL INVESTIGATOR: Henry J. Thompson, Ph.D.

CONTRACTING ORGANIZATION: Colorado State University
                                           Fort Collins CO 80523

REPORT DATE: December 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                         Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                                          Distribution Unlimited

REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
01-12-2006	Final	01 Aug 01 – 30 Nov 06

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Selenium and Breast Cancer Chemoprevention	
	5b. GRANT NUMBER
	DAMD17-01-1-0124
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Henry J. Thompson, Ph.D.	
	5e. TASK NUMBER
E-Mail: henry.thompson@colostate.edu	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Colorado State University Fort Collins CO 80523	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The primary objective of this project was to determine whether selenium supplementation affected candidate markers of breast cancer risk in a cohort of women at elevated risk for breast cancer. The intermediate biomarkers being studied were: indicators of oxidative damage to cellular macromolecules such as DNA and lipid, indicators of IGF metabolic status, and cellular indicators of breast cancer risk. We conducted a randomized, placebo-controlled, double-blind chemoprevention trial with 150 participants (75 subjects per arm) using a placebo tablet or a tablet containing 200 μg high-selenium brewer's yeast per day, given for a duration of one year. The form and dose of selenium that was being used has been reported to reduce cancer incidence and mortality in lung, prostate, and colon. Blood and urine were collected at baseline, and after 6 and 12 months of intervention. The feasibility of obtaining breast epithelial cells via nipple aspiration at baseline and the end of the intervention was assessed. Plasma selenium and glutathione peroxidase activity were evaluated in addition to pill counts and self report as markers of compliance.

15. SUBJECT TERMS
Breast Cancer, Prevention, Chemoprevention, Selenium, Oxidative Damage, IGF-1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
U	U	U	UU	12	

Table of Contents

Introduction

The primary objective of this project was to determine whether selenium supplementation affected candidate markers for breast cancer risk in a cohort of women at elevated risk for breast cancer. The intermediate biomarkers studied were: indicators of oxidative damage to cellular macromolecules such as DNA and lipid, indicators of IGF metabolic status, and cellular indicators of breast cancer risk.

Body of Report

Approved Statement of Work

We are conducting a randomized, placebo-controlled, double-blind chemoprevention trial with 150 participants (75 subjects per arm) using a placebo tablet or a tablet containing 200 µg high-selenium brewer's yeast per day, given for a duration of one year. Blood and urine are being collected at baseline, and after 6 and 12 months of intervention. Efforts are being made to obtain breast epithelial and/or breast fluid via nipple aspiration using a modified breast pump. This procedure is performed at baseline and the end of the intervention. Randomization will be in 15 blocks of 10 subjects each.

1. Year 01
   a. Final development of project materials including Web-based randomization program, data entry screens, data quality assurance procedures, project databases.
   b. Obtain all supplements.
   c. Initiate recruitment and enter 3 blocks of 10 subjects.
   d. Schedule follow-up visits.
   e. Institute monthly patient follow-up.
   f. Ongoing collection and analyses of biological samples.
   g. Enter results into databases.
   h. Submit progress report.
2. Years 02-03
   a. Enter remaining subjects into the study and continue follow up, sample collection and analyses. Goal is 8 blocks of 10 in year 02 and 4 blocks of 10 in year 03.
   b. Submit progress reports.
3. Year 04
   a. Complete follow up and the collection and analysis of all samples.
   b. Evaluate all data.
   c. Summarize findings for publication and submit final report.

No Cost Extension A one year no cost extension was requested and approved.
   The project ended December 31, 2006.

Acronym for Study We refer to this project as the **ENRICH** study.

The study was conducted over four years. Accrual began in August of 2002, and ended in July, 2005. The last subject completed the study protocol in July, 2006. A total of 162 subjects enrolled, 111 completed the first clinical visit, 98 completed the 2nd visit, 94 the 3rd ; 94 completed the entire study.

During the course of the study each participant completed several questionnaires and three physical exams. Nipple aspirations were done on a voluntary basis. Twenty-six of 59 attempted at baseline were successful; thirteen of 25 at visit 3 were successful. The initial questionnaires elicited information about demographics, past medical history, supplement use, current medications, and dietary habits. The participants also provided samples of blood and urine (samples collected at home and frozen and a fresh sample) at each of the 3 clinic visits. At the first visit each participant was supplied with multivitamin tablets and study tablets to last until her next visit in approximately 6 months, when taken once daily. Compliance was assessed by measuring plasma selenium levels and pill counts. Gail score and breast density were used to stratify subjects to attain balance across the placebo and intervention arms on these two important breast cancer risk variables.

Table 1: Participants who completed the study by visit and study arm.

Study Arm	Enrollment	Visit1	Visit 2	Visit 3	**Dropout Rate***
Selenium	134	55	48	47	**15%**
Placebo		56	50	47	**16%**
Not Randomized	28				
Total	162	111	98	94	**15%**

*Dropout rate computed based on the subjects who completed their first clinic visit.

Overview of Statistical Methods

Maximum likelihood (ML) estimates of a multivariate repeated measures model using all available data was used for the analysis of the primary outcomes (levels of DNA oxidation, lipid peroxidation, IGF1, IGFBP3). This approach is conceptually identical to multivariate analysis of variance (MANOVA) but avoids the case-wise deletion of subjects with missing assessments, and relaxes the assumption that missing data are missing completely at random (MCAR). The model provides unbiased estimates under the less restrictive assumption that missing data are missing at random (MAR). The ML estimates are based on a repeated measures model of time by group:

$y_{ij} = \beta_0 + \beta_1 tG$ where y_{ij} is the outcome measure for the ith subject in the jth randomization group; j={1,2}; t ={0, 1, 2}; G=A if the subject is in the Selenium group, B otherwise. Analyses were done on the log transformation of the DNA damage, 8-ISO-PGF$_{2\alpha}$,SOD, GPx, IGF-1, and IGFBP3 to stabilize the variance of the data.

Baseline differences in cohort characteristics across randomization groups were evaluated using a chi-square test for homogeneity of proportions for categorical variables, t-tests or two-group t-tests on the log transform for continuous variables, depending on their distribution.

Accrual and Dropout

Participation in this Institutional Review Board approved study was completely voluntary. Participants were recruited during the regularly scheduled clinical risks at our high risk breast clinic referred to as BreastWatch. One hundred sixty-two subjects completed the eligibility questionnaire and were enrolled in the study, but only 111 subjects completed the first clinic visit. Thirteen subjects withdrew after visit 1, and another 4 after visit 3. Loss to follow-up was 15% in each treatment arm. There was no significant difference regarding age, Gail score,

breast density or randomization group between those who completed the study and those who dropped out. The clinical data, DNA damage, 8-isoprostane F2 alpha (8-ISO-PGF$_2$, superoxide dismutase (SOD), glutathione peroxidase (GPx), insulin like growth factor-1 (IGF-1), and insulin like growth factor binding protein 3 (IGFBP3), showed no difference between those who completed only visit 1 and those with data for all 3 visits.

Cohort Characteristics

The study participants were predominantly white (96%). Their median age was 49 years (range = 22 to 78). Ninety-seven percent of the participants had more than 12 years of education, 79% reported at least a college degree. They reported consuming an average of 4 ± 2.8 (mean ± SD) servings of vegetables and fruit daily, and had measured BMI of 24 ± 4.5.

No baseline characteristics were significantly different by study group. Variables tested were age, race, education, daily servings of vegetables and fruit, plasma selenium, body mass index (BMI), 8-ISO-PGF$_{2\alpha}$, SOD, GPx, IGF-1, and IGFBP3. GAIL score and breast density were used to stratify the randomization; their means ± SD at baseline were 3.12 ± 1.90 and 54.7% ± 15.9%, respectively.

Compliance Marker Data

Pill Count Overall compliance measured by pill count was high; 95% in the placebo group and 96% in the Selenium group. Biological markers for selenium supplementation, i.e. plasma selenium were also assessed and are reported below**.**

Plasma Selenium Levels A clear difference in mean plasma selenium level by study arm with very little overlap was maintained at 6 and 12 months; table 2 and Figure 1 below show the similarity in plasma selenium levels at all visits in the Placebo (PBO) group and the differences at Visits 2 and 3 for the Selenium group (Se). The medians are joined by a red line, and red stars identify data points that lie to the left of the 5th percentile or the right of the 95th for each box.

Figure 1. Plasma Selenium Levels at visits 1, 2, and 3 by study arm. Medians are joined by a red line; points outside the 5th or 95th percentiles are displayed as a red star.
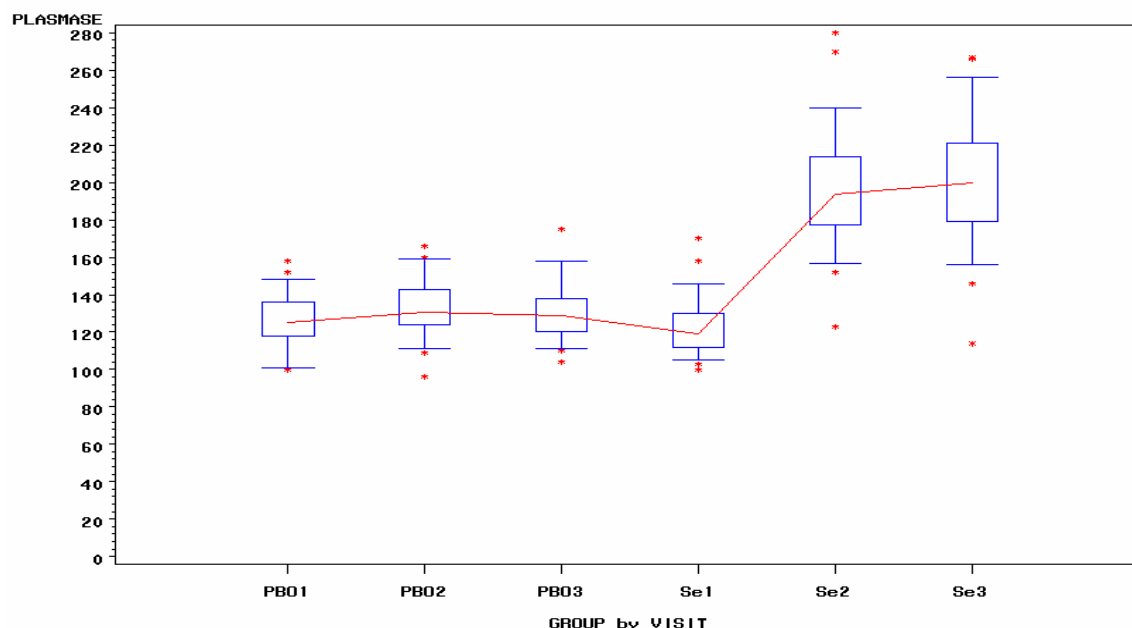
Table 2 Plasma Selenium Levels at visits 1, 2, and 3 by study arm

	Treatment Group	Mean ± SD		
		Baseline (N=111)	6 months (N=98)	12 months (N=93)
Plasma Se	Selenium	122.28±13.99	195.60±29.35	203.00±34.11
	Placebo	126.02±12.87	132.30±14.58	130.78±14.66

Oxidative Damage Endpoints

8-ISO PGF$_{\alpha 2}$ 8-ISO was not significantly lower in the selenium group at either visit 2 (p=0.46) or visit 3 (p=0.70).

DNA damage DNA damage was not significantly lower in the selenium group at either visit 2 (p=0.51) or visit 3 (p=0.54).

GPx, SOD Median GPx was higher in the Selenium intervention group than in the placebo group at 6 months by 8.2 % (p= 0.04) and at 12 months by 8.7 % (p=.03); the difference between groups at 6 and 12 months for SOD was not significant (p=.93 and p=.95 respectively).

IGF-1, IGFBP-3 Neither measure responded to the selenium intervention; the differences were not significant at either visit 2 (p=0.68) or visit 3 (p=0.96).

**Table 3 Median Values for Outcome Measures
at Baseline and Follow-up (median (pctl 25, pctl 75))**

Outcome Measure	Tx Group	Baseline (N=93)	6 months (N=93)	p	12 months (N=93)	p
8-ISO (pg/ug creatinine)	Se	0.49 (0.31, 0.72)	0.54 (0.38, 0.73)	0.46	0.43 (0.30, 0.66)	0.70
	Placebo		0.49 (0.34, 0.69)		0.50(0.35, 0.75)	
DNA damage (au/cell)	Se	46.6 (35.2, 55.00)	45.4 (39.1, 52.1)	0.51	36.2 (22.8, 49.5)	0.54
	Placebo		50.3 (39.9, 57.2)		31.4 (30.7, 36.4)	
IGF1 (ng/ml)	Se	238.3 (184.2, 305.5)	266.1(193.7, 306.1)	0.76	237.3 (181.0, 307.0)	0.81
	Placebo		242.1 (176.5, 295.3)		220.7 (176.0, 298.2)	
IGFBP3 (ng/ml)	Se	4527.70 (3954.50, 5388.50)	4402.10(3819.70, 5332.50)	0.68	4213.50(3762.00, 4993.40)	0.96
	Placebo		4251.45(3992.30,5233.60)		4188.15(3857.90, 5098.80)	
SOD (U/mL)	Se	3.87 (3.08, 4.67)	3.95(3.24, 4.98)	0.98	3.77(3.27, 4.73)	0.99
	Placebo		4.23(3.26, 4.82)		4.17(3.51, 4.82)	
GPx(nmol/min/ml)	Se	103.53 (82.90, 115.43)	121.04(105.03, 137.79)	0.04	121.49(112.34, 133.94)	0.03
	Placebo		111.82(91.15, 127.72)		111.72(96.51, 134.17)	

Post Hoc Analyses Based on our recently published work indicating that individuals with high levels of lipid peroxidation respond to antioxidant interventions to a greater extent, this post hoc analysis was performed.

8-ISOPGF$_{\alpha 2}$ by Quartiles There was no evidence of a differential effect of the intervention by baseline quartile of 8-ISOPGF$_{\alpha 2}$ for those in the upper quartile at baseline is marginally lower at 12 months in both groups, while for those in the lower quartile it is marginally higher in both groups, suggesting regression to the mean.

EPG by Quartile, Visit, and Group
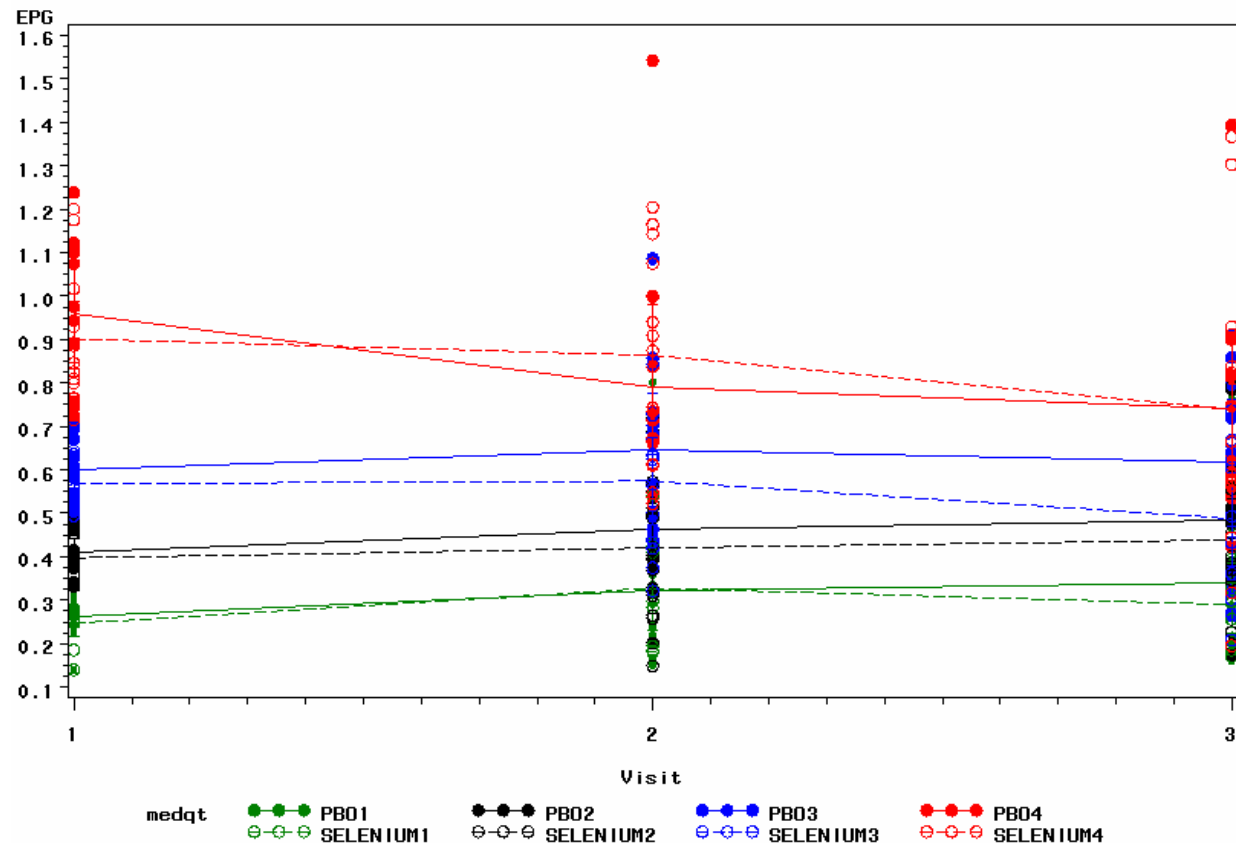
Other cancer risk biomarkers As noted in the annual project reports, we found that nipple aspirate fluid (NAF) could be collected from only about half of women who agreed to provide NAF and that there was limited cellular content in the material that was collected. For this reason we decided to initiate efforts to identify a serum proteomic profile that could be used to assess changes in breast cancer risk. We decided that the best way to do this was to engage in a collaborative effort with Paul Lampe, director of the proteomics mass spectrometry core in the Division of Cancer Prevention at Fred Hutchinson Cancer Research Center. We judged that efforts to identify proteomic markers have met with limited success because most investigators have not taken advantage of the power of pre-clinical models for method development and proof-in-principle experiments. While this work WAS NOT supported by this project, we remain committed to evaluating the serum and/or NAF samples collected once a validated assessment

profile is identified and can be performed via ELISA so as to insure the accuracy of marker quantification. The following is a brief description of what has been accomplished to date.

An experiment was designed to determine whether a proteomic profile could be identified in serum that can be used to quantify cancer risk and that correlates with disease burden during pre-malignant and malignant stages of breast carcinogenesis. The hypothesis underlying this investigation was that there is a "field effect" in the mammary gland of carcinogen treated animals that precedes histologically detectable disease. This will result in changes in the serum proteome. Furthermore, as pre-malignant and malignant pathologies are detected this will further alter the serum proteome. We seek to determine if the hypothesized effects can be detected. Of particular interest is whether the proteomic profile changes qualitatively with disease state and/or whether changes are in amount of protein rather than its type.

A chemically induced, rapid emergence model for breast cancer was used for this investigation. The temporal sequence of occurrence of premalignant and malignant mammary pathologies has been established and mammary carcinomas are observed in the majority of animals within 35 days following carcinogen administration. In this experiment, female Sprague Dawley rats are injected at 21 days of age with 50 mg 1-methyl-1-nitrosourea (MNU) per kg body weight or the saline solvent in which MNU is dissolved.   At 14, 21, 28, 35, days post carcinogen, 5 saline and 15 MNU-injected rats were euthanized and mammary gland whole mount were prepared for quantification of disease burden. At necropsy, blood was obtained via retro orbital sinus bleeding and subsequently processed to recover serum. Serum was aliquoted (50 ul/cryovial) and stored at -80C until it was analyzed.   Large, abundant proteins were removed from the serum via acetonitrile precipitation to obtain the "peptidome". MALDI-TOF spectra were obtained on an Applied Biosystems Voyager-DE PRO Biospectrometer and analyzed using peak picking computer algorithms and logistic regression models.

MALDI-TOF analysis was performed on sera taken from control and carcinogen-treated at each necropsy time point. Three peaks (m/z values of 1200, 1228, and 1743) revealed a monotonic change in the intensity difference between the treated and untreated rats over weeks 2, 3, 4, and 5. The statistical significance of the intensity difference of these peaks also increased monotonically over these weeks. The intensity difference of these peaks between the treated and untreated rats was found to be statistically significant in the fourth and fifth weeks. We are currently determining the identity of the peaks. Although further validation is necessary, our MS methods are consistent with the idea that serum biomarkers are altered with disease progression during pre-malignant and malignant stages of mammary carcinogenesis. This work has the potential to identify biomarkers for breast cancer risk assessment.

Key Research Accomplishments
- The selenium intervention used in this project has been reported to reduce cancer mortality and it is generally presumed that selenium mediates this effect via the induction of glutathione peroxidase activity and the consequential effect of the active form of this protein on antioxidant status. We found no evidence to support this hypothesis. This is important information for individuals at increased risk for breast cancer given the strong evidence that uncontrolled cellular oxidation is involved in the progression of this disease process. Based on the data obtained in this project, selenium supplementation is not expected to affect antioxidant status. While in some respects this represents a negative finding, we judge that the medical community should be made aware of this information; it is being written up as a brief communication for publication

in an appropriate medical journal.

- As outlined in the grant application, there is a small and controversial literature about whether selenium supplementation alters the metabolism of insulin-like growth factor-1 (IGF-1) and its dominant binding protein, IGFBP-3. Elevated IGF-1 relative to its binding protein is considered to indicate increased risk for breast cancer. We found no evidence to support the hypothesis that IGF-1 or IGFBP-3 metabolism was affected by the selenium intervention. While this is a negative finding, it is important information to share with the research community; these data are being written up as a brief communication for publication in an appropriate medical journal.

Reportable Outcomes
- Supporting intervention materials were developed and tested (when appropriate).
- The project database was completed
- The intervention phase of the project was completed.
- The measurements of biomarker outcomes and data evaluation were completed.
- Data are summarized and ready for submission for publication in peer-reviewed medical journals.

Conclusions A strong body of evidence from preclinical models indicates that selenium supplementation has the potential to reduce breast cancer risk, yet the majority of selenium intervention trials are being conducted in men only. This investigation of the effects of the same selenium intervention reported to reduce prostate cancer mortality in men failed to identify a mechanistic basis for hypothesizing that selenium supplementation will reduced breast cancer risk in women. Currently, there is not a strong justification for proposing an intervention trial in women at risk for breast cancer.

References
1. Clark,L.C., Combs,G.F., Jr., Turnbull,B.W., Slate,E.H., Chalker,D.K., Chow,J., Davis,L.S., Glover,R.A., Graham,G.F., Gross,E.G., Krongrad,A., Lesher,J.L., Jr., Park,H.K., Sanders,B.B., Jr., Smith,C.L., and Taylor,J.R. (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group [see comments] [published erratum appears in JAMA 1997 May 21;277(19):1520]. *JAMA*, **276**, 1957-1963.
2. Clark,L.C., Combs,G.F., Jr., Turnbull,B.W., Slate,E.H., Chalker,D.K., Chow,J., Davis,L.S., Glover,R.A., Graham,G.F., Gross,E.G., Krongrad,A., Lesher,J.L., Jr., Park,H.K., Sanders,B.B., Jr., Smith,C.L., and Taylor,J.R. (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *JAMA*, **276**, 1957-1963.
3. Clark,L.C., Dalkin,B., Krongrad,A., Combs,G.F., Jr., Turnbull,B.W., Slate,E.H., Witherington,R., Herlong,J.H., Janosko,E., Carpenter,D., Borosso,C., Falk,S., and Rounder,J. (1998) Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. *Br.J.Urol.*, **81**, 730-734.
4. Clark,L.C. and Marshall,J.R. (2001) Randomized, controlled chemoprevention trials in populations at very high risk for prostate cancer: Elevated prostate-specific antigen and high-grade prostatic intraepithelial neoplasia. *Urology*, **57**, 185-187.

5. Combs,G.F., Jr., Clark,L.C., and Turnbull,B.W. (1997) Reduction of cancer mortality and incidence by selenium supplementation. *Med.Klin.*, **92 Suppl 3**, 42-45.
6. Combs,G.F., Jr., Clark,L.C., and Turnbull,B.W. (1997) Reduction of cancer risk with an oral supplement of selenium. *Biomed.Environ.Sci.*, **10**, 227-234.
7. Nelson,M.A., Porterfield,B.W., Jacobs,E.T., and Clark,L.C. (1999) Selenium and prostate cancer prevention. *Semin.Urol.Oncol.*, **17**, 91-96.
8. Duffield-Lillico,A.J., Reid,M.E., Turnbull,B.W., Combs,G.F., Jr., Slate,E.H., Fischbach,L.A., Marshall,J.R., and Clark,L.C. (2002) Baseline Characteristics and the Effect of Selenium Supplementation on Cancer Incidence in a Randomized Clinical Trial: A Summary Report of the Nutritional Prevention of Cancer Trial. *Cancer Epidemiol Biomarkers Prev*, **11**, 630-639.

Appendix A

Patient Characteristics by Treatment Group at Baseline

Measure	Mean (SD)		
	Placebo N=56	Selenium N=55	*p*
Age	50.23 (10.51)	49.33 (7.61)	0.60
Gail Score	3.08 (1.88)	3.15 (1.93)	0.85
Breast Density (%)	55.0 (16.6)	54.4 (15.4)	0.83
Plasma Selenium ng/ml	126.07 (12.98)	122.28 (13.99)	0.14
Average Daily Servings V&F	4.27 (3.21)	3.90 (2.24)	0.52
BMI (kg/m^2)	23.87 (4.19)	25.04 (4.73)	0.17

Patient Characteristics by Withdrew Status at Baseline

Measure	Mean (SD)		
	Withdrew N=17	Completed N=94	*p*
Age	47.29 (10.08)	50.23 (8.96)	0.27
Gail Score	3.41 (2.54)	3.07 (1.77)	0.60
Breast Density (%)	54.7 (18.4)	54.7 (15.6)	0.99
Plasma Selenium (ng/ml)	126.50 (10.93)	123.82 (13.96)	0.39
Average Daily Servings V&F	3.50 (0.40)	4.10 (2.79)	0.21
BMI (kg/m^2)	23.60 (4.99)	24.60 (4.39)	0.45